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Research Article

QUANTITATIVE ANALYSIS OF *GYRA* AND *GYRB* KNOWN MUTATIONS IN DRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* STRAINS TREATED WITH OFLOXACIN

PUSHPARAJU RAMASAMY[1], VIGNESH SOUNDERRAJAN[2], SHAKILA HARSHAVARDHAN[1]*

[1]Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu, India. [2]Department of Zoology, G.T.N. Arts College, Dindigul, Tamil Nadu, India. Email: mohanshakila@yahoo.com

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ABSTRACT

Objective: The aim of the study is to measure the minimum inhibitory concentrations (MICs) of ofloxacin antibiotic from *gyrA* and *gyrB* mutations present in fluoroquinolones (FQs) resistant strains of *Mycobacterium tuberculosis* (MTB) and to further concentrate the potential association between gene mutations and phenotypic resistance based on their MICs.

Methods: Different levels of ofloxacin MICs levels were detected in 31 archived multi drug-resistant MTB isolates showing *gyrA* mutations in codon at A90V, S91P, D94A, D94N/Y, D94 G, and D94H and two *gyrB* probes (N538D and E540V). The MIC determinations were made using the 1% proportion method.

Results: Genotypic assay detected 32 mutations in the *gyrA* (n=29) and gyrB (n=3) genes among the 31 FQs resistant isolates. Most frequently seen in *gyrA* mutations at codon D94G (n=16; 50%), these mutations had a clearly elevated MIC level from 2 to 16 µg/ml, that was phenotypically resistant. The A90V mutation region consistently exhibited the lowest levels of ofloxacin resistance, with three out of eight (37.50%) of these isolates had a MIC of <2 µg/ml. In addition, a further strain of S91P mutations for MIC was determined to be less than the critical concentration (CC). These low levels of resistance have been detected in a phenotypic manner which is noticeable in the study. Furthermore, fewer mutations in codons at D94A, D94N/Y were identified. Three wild-type absent isolates from *gyrB* QRDR were identified and the MIC of those strains for ofloxacin was lower than the critical cutoff value.

Conclusion: Based on the results, it is shown that different resistance mutations were associated with different levels of MICs and the current concentration for MGIT will be lowered from 2 µg/ml to 1 µg/ml for the ofloxacin drug.

Keywords: *Mycobacterium tuberculosis*, *gyrA*, *gyrB*, Minimum inhibitory concentration, ofloxacin, mutations.

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INTRODUCTION

Drug-resistant tuberculosis (TB) is a major public health concern and the global burden of TB is high. There is a growing concern of increase and distributions of multi and extensively drug-resistant TB (MDR/XDR-TB) makes it increasingly important that drug susceptibility testing (DST) of *Mycobacterium tuberculosis* (MTB) strains produce results that are clinically significant and technically reproducible [1]. Currently available laboratory diagnostic modalities for the detection of drug-resistant TB depend on either phenotypic DST or genotypic DST methods. Phenotypic DST relies on the testing of a single, critical concentration (CC) of each antibiotic agent and it can only differentiate sensitive or resistant to phenotypically wild type (pWT) or phenotypically non-wild type (pNWT) of *MTB complex* (MBTC) strains [2]. Molecular diagnostic assay, such as the Xpert MTB/ RIF and line probe assays, improves the identification of drug resistance for MDR and XDR-TB, but it can detect only limited numbers of specific mutations conferring resistance to the given drugs [3]. Unfortunately, both molecular drug detection and phenotypic DST at CCs are qualitative, giving rise to clinical uncertainties regarding the optimal drug dosing required to treat patients who may have different levels of resistance to the different TB treatment drugs.

Fluoroquinolone (FQs) class antibiotics (ofloxacin, levofloxacin, moxifloxacine, and gatifloxacin) are second-line TB drugs and currently play an important role in the treatment of TB, particularly MDR-TB. FQs bind and inhibits mycobacterial topoisomerase II (DNA gyrase) and topoisomerase IV, enzymes involved in bacterial viability, resulting in cell death. The development of FQs resistance mutations in *MTB* is due to the quinolone resistance-determining (QRDR) regions, *gyrA* or *gyrB* [4]. Several previous studies reported that the existence of these various mutations in *MTB* genes is correlated with different levels of fluoroquinolone resistance [5-7]. While there is some variation in some mutations, these results are low-level FQs resistance and others may lead to a higher level of resistance [8]. We have predicted that different resistant mutants have been associated with different MIC levels. In this study, quantitative phenotypic DST was performed with MIC measurements for ofloxacin drug from *gyrA* and *gyrB* mutations present in fluoroquinolone resistant MDR-TB strains. In addition, the possible correlation between gene mutations and phenotypic resistance to ofloxacin was further evaluated on the basis of their MICs.

MATERIALS AND METHODS

Strains and study population

This is a retrospective study. Thirty-one archived isolates were FQ drug-resistant among 191 suspected XDR-TB identified and FQ mutations were genotypically detected. These culture isolates were selected for drug susceptibility testing with WHO-approved CCs and tested at different drug concentrations for MICs. The study was carried out on archived isolates.

Detection of fluoroquinolones resistance

GenoType MTBDRsl VER 2.0 assay was performed for the detection of fluoroquinolone resistant mutations of MDR-TB, according to the manufacturer's instructions (Hain Lifescience GmbH, Nehren, Germany) [9]. The MTBDRsl experiment is based on three-step DNA strip technology: Extraction of DNA, a multiplex – polymerase chain

reaction (PCR), amplification, and reverse hybridization. DNA isolation was performed on decontaminated smear positive sputum samples or liquid culture isolates using a GenoLyse (R) kit (HainLifescience, Nehren, Germany). For multiplex PCR amplification, a volume of 5 µl of isolated DNA was used utilizing biotinylated primers. The line probe assay (LPA) assay was considered as test valid; only when hybridization bands were obtained for conjugate control, the amplification control an MTB complex-specific control, bands conjunction along with the targeted gene loci controls. The DNA strip genotypic assay contains 12 probes for the fluoroquinolones antibiotic group with the exception of amplification, hybridization, and gene loci control probes. The resistance of FQ was detected using seven probes (A90V, S91P, D94A, D94N/Y, D94 G, and D94H) for *gyrA* and two *gyrB* probes (N538D and E540V). The presence of all wild-type (WT) signal and the absence of mutation (non-developed) bands are designated as susceptibility. The absence of WT probes (undefined mutations) or the development (presence) of the specific mutation signal (defined mutations) is associated with specific genes on the hybridization strip, and the result was interpreted as resistance to the FQ drug [10].

Phonotypic MGIT DST

The quantitative phenotypic DST was performed using the BACTEC MGIT960 (BD Diagnostic Systems, Sparks, USA) instrument. Genotypically confirmed that FQ resistant isolates were sub-cultured and performed by conventional DST with MGIT960 tube. CCs were used for DST in the MGIT 960 system using a critical proportion of 1% [11] for FQs drugs as ofloxacin was 2.0 µg/ml, as recommended by the WHO [2].

Drug preparation for MGIT DST

The stock solution of ofloxacin (Sigma-Aldrich) antibiotic compound was prepared by dissolving the drug in 0.1N NaOH. Required drugs were filtered, further diluted in sterile distilled water, and stored at −80°C. Preparation of the test inoculum, followed by inoculation and further incubation was performed as per manufacturer's instructions (Becton Dickinson Diagnostic System, Sparks, MD). FQ resistant strain and pan susceptible H37Rv strain were tested by MGIT liquid DST as a quality control.

MIC testing

To fully characterize the phenotypic resistant pattern of the *gyrA* and *gyrB MTB* isolates, different MICs were selected. The MIC determinations were made using the BACTEC MGIT 960 system. The MIC was described as the lowest antibiotic concentration which had shown less than 100 growth units (GUs) when the 1:100 diluted controls reached 400 growth units. For the estimation of MICs of ofloxacin drug in *MTB* strains, test concentrations below the two CCs of 0.5 µg/ml, 1.0 µg/ml, and three above CC of 4.0 µg/ml, 8.0 µg/ml, and 16.0 µg/ml were used [7,8,12]. For the reference *MTB* strain, ofloxacin was tested in two-fold dilutions.

RESULTS

A total number of 31 FQ resistance isolates were identified among 191 MDR-TB in this study, using the GenoType MTBDR*sl* VER 2.0 assay. There were 31/191 (16.23%) isolates resistant to FQs as tested by genotypic DST. GenoType MTBDR*sl* detected 32 mutations in the *gyrA* and *gyrB* genes among the 31 FQs resistant isolates. Table 1 summarizes the distribution of mutations. The large number of mutations, *gyrA* MUT3 18/31 (58.06%), were found most frequently at *gyrA* codons at D94G (*gyrA* MUT3C -16 No's), D94A (*gyrA* MUT3A- 1 No's) and D94N/Y (*gyrA* MUT3B -1 No's). Other *gyrA* mutations observed by the assay were at codon A90V (*gyrA* MUT1; 8/31; 25.8%) and at codon S91P (*gyrA* MUT2; 3/31; 9.67%). All fluoroquinolone resistant mutations were previously reported [10,13-15]. One isolate among these exhibited harbored two mutation bands involving codons 94 and 91 and other 2 numbers amino acid codon position at 94, WT/*gyrA*MUT3C. Defined mutations that were not detected in the *gyrB* region (mutations, i.e., N538D and E540V) but undefined mutation, that is, wild type absence was detected among MDR-TB tested population. The contribution of undefined mutation in *gyrB*QRDR to FQ resistance was 3 (9.67%) numbers only as previously reported [10,15]. Heteroresistance pattern in *gyrA* gene has been observed in 3/31 (16.5%) of FQ - resistant isolates, as previously shown [10].

The MICs of ofloxacin tested against the *MTB* isolates carrying gyrase mutations within the QRDR and 28 strains with mutations in the QRDRs of *gyrA* and three strains without mutations (wild type deletion) in the QRDR *gyrB*. MIC was clearly distinct between susceptible and resistant strains. The results of MIC for *gyrA and gyrB* mutations are summarized in Table 1 and Fig. 1. We detected 15 isolates, harboring the D94G mutations, all of which were found to have an OFX MIC of 2.0–16.0 µg/ml. Eight of A90V mutation isolates, MIC was observed at low to high levels of resistance [12,13]. For these, 5 numbers equal to or greater than the CCs at 2.0 µg/ml, while 3 numbers below the CCs. Two S91P mutations had MICs of 1.0µg/ml and 2.0µg/ml. D94A mutations had MICs of 4.0µg/ml and D94N/Y had MICs of 2.0µg/ml. One isolate with two mutations presents, S91P, D94G, and a higher MIC level of 8.0µg/ml for OFX. Three wild type isolates from the *gyrB* gene were MIC of two strains of 1.0 µg/ml and one remaining was 0.5 µg/ml, similar findings were reported [5]. Overall, 24 (77.41%) of the isolates 28 with known *gyrA* resistant mutations were phenotypically resistant to ofloxacin at the CC of equal to 2.0 or above up to 16µg/ml and remaining 4 numbers lowered than the current CC whereas three genotypically wild type *gyrB* resistant isolates were lower than CC, where these results are considered as phenotypically susceptible.

DISCUSSION

In this study, MIC determination using the MGIT is an excellent traditional DST method and is relatively rapid, up-front, and provides quantitative data on susceptibility to OFX drug, making it easier for therapeutic decision-making and therapeutic drug monitoring to maximize protocol effectiveness and reduce toxicity. The molecular genotyping assay has been shown to be a sensitive, accurate, rapid, and feasible method for MTB drug susceptibility testing [16]. Each of these *MTB* isolate was shown to contain a resistance mutation in either the *gyrA* or the *gyrB* genes in the QRDR. The presence of mutations in the fluoroquinolone gene was, therefore, correlated with the OFX resistance at the CCs described above.

Table 1: Mutations in *gyrA* & *gyrB* and MICs profiles of MTB strains determined by proportion method

Target region	Mutation(s) detected	No. (%) of mutations	Ofloxain MIC µg/ml					
			0.5	1	2	4	8	16
gyrA MUT1	A90V	8 (25.80%)		3	1	3	1	
gyrA MUT2	S91P	2 (6.45%)		1	1			
gyrA MUT3A	D94A	1 (3.22%)				1		
WT/gyrA MUT3C	D94G	2 (6.45%)				1	1	
gyrA MUT3B	D94N/Y	1 (3.22%)			1			
gyrA MUT3C	D94G	13 (41.93%)			2	6	3	2
WT/gyrA MUT2 and 3C	S91P, D94G	1 (3.22%)					1	
gyrB WT1 absent	536 -541	3 (9.67)	1	2				
		Total	1	6	5	11	6	2

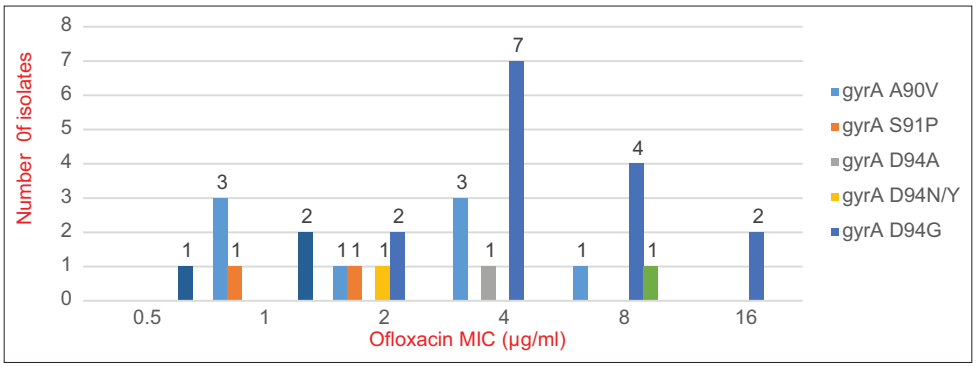

Fig. 1: Ofloxacin MIC results stratified by known resistance mutations in the QRDR region of *gyrA* or *gyrB* mutations

The prevalence of *gyrA* mutations in clinical isolates from quinolone-resistant TB varies across countries, ranging from 64.5% to 94.1% [17]. In this study, the relatively high prevalence of mutations in QRDR-*gyrA* region was found to be 90.34% of the isolates studied and the most frequent mutations occurred in *gyrA* at amino acid codon at 94 (58.06%). These 94 codon (D94A, D94N/Y, and D94G) mutations were shown to be associated with a clearly elevated MIC level from 2 to 16 µg/ml, which was phenotypically resistant. Compared to mutation isolates elsewhere in the *gyrA* QRDR, MTB isolates with A90V mutation region exhibited consistently the lowest levels of resistance to OFX, with 3 of 8 (37.50%) of these isolates having a MIC of < 2 µg/ml (Table 1). Others have previously reported that similar mutations inside the *gyrA*QRDR results in various FQ MICs [6,7,12,13,16,18]. These low levels of resistance detected in the population of these mutants are considered to be phenotypically susceptible which is noteworthy of the study. Furthermore, one of the isolates bearing S91P mutations would be considered to be susceptible to OFX, the MIC was determined to be less than CC, and the remaining strain was resistant. One double mutation of the isolates S91P and D94 G and ofloxacin MIC was 8 µg/ml; the multiple mutations were associated with the high degree of resistance. We analyzed MICs associated with each resistant *gyrA* codons in this study and interestingly, certain *gyrA* mutations, A90V and S91P were found even in low-level MIC, compared to the current ofloxacin CC level.

In addition, mutations in the *gyrB* gene are less commonly associated with FQ resistance in MTB. Undefined mutations within the codons *gyrB*QRDR associated with ofloxacin susceptible were identified by phenotypically in all three (9.67%) isolates with a MIC lower than the critical cutoff value, and known to be implicated in a phenotypically low-level of FQ resistance as observed in our work and also in others [5,19]. Von Groll *et al.* reported that the mutation in *gyrB* was linked with resistance to other drugs of the quinolone class, such as moxifloxacin (MFX) and gatifloxacin (GFX), but interestingly not ofloxacin. It was speculated that the absence of resistance to ofloxacin might be due to a more interactive of *gyrB* with common structures of MFX and GFX, such as the C-8 methoxy derivative or the C-7 methyl piperazine ring which is absent in ofloxacin[18].

CONCLUSION

We compared genotypically identified drug-resistant mutations linked with MICs and quantitatively observed that different mutations showed in different levels of drug resistance. In fact, in the present study, certain *MTB* isolates associated with mutations are unable to detect the corresponding resistance by the phenotypic gold standard method as liquid MGIT DST due to a possible reason for high concentration of drug testing. The MICs for these subjected drugs were often around the CC and increased probability of misclassified as susceptible to ofloxacin with MGIT 960 system. Based on the data from this study, we suggest, the current CC for MGIT should be lowered from 2 µg/ml to 1 µg/ml for ofloxacin drug and phenotypic DST, in line with the previous observations [19,20]. Even more investigation of these issues through further studies correlating the low-level resistance to ofloxacin is needed to determine the CC testing.

AUTHOR'S CONTRIBUTIONS

All the work carried out by the authors and the same in the preparation of the manuscript.

CONFLICTS OF INTEREST

No conflicts of interest are declared by the authors.

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